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(71) Applicant (for all designated States except US): THE UNIVERSITY OF BRITISH COLUMBIA [CA/CA]; University Industry Liaison Office, IRC Building, Room 331, 2194 Health Sciences Mall, Vancouver, British Columbia V6T 1Z3 (CA).

[CA/CA]; 4014 West 21st Avenue, Vancouver, British Columbia V6S 1H9 (CA). MONTANER, Julio [CA/CA]; 3230 West 24th Avenue, Vancouver, British Columbia V6L 1R9 (CA). O'SHAUGHNESSY, Michael, V. [CA/CA]; 27425 110th Avenue, Maple Ridge, British Columbia V2W 1P5 (CA).

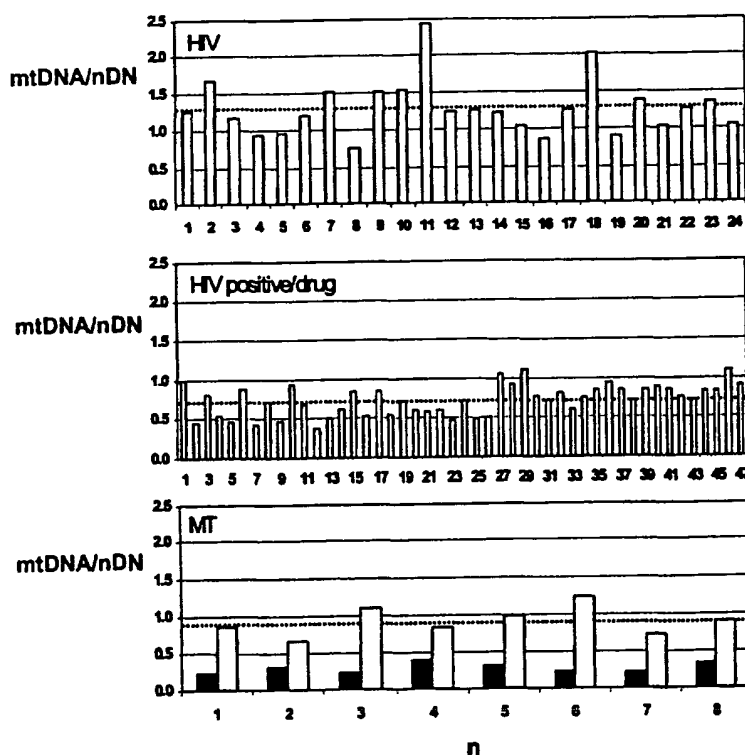
(74) Agents: KINGWELL, Brian, G. et al.; Smart & Biggar, Box 11560, Vancouver Centre, Suite 2200, 650 W. Georgia Street, Vancouver, British Columbia V6B 4N8 (CA).

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(72) Inventors; and
(75) Inventors/Applicants (for US only): COTE, Helene

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(54) Title: PHARMACOLOGICAL APPLICATIONS OF MITOCHONDRIAL DNA ASSAYS



(57) Abstract: This invention provides assays to determine the relative amount of mitochondrial DNA in a subject, such as a subject undergoing drug treatment. The subject may for example be a human patient undergoing treatment for an HIV infection with a nucleic acid precursor such as a nucleoside or nucleotide analogue. The assays of the invention may include PCR assays, such as semi-quantitative or quantitative PCR involving the co-amplification of a mitochondrial sequence and a reference sequence, such as a genomic sequence. Information from such assays may be evaluated to provide a ratio of mitochondrial DNA to nuclear DNA in the cells of the subject.

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PHARMACOLOGICAL APPLICATIONS OF MITOCHONDRIAL DNA ASSAYS

FIELD OF THE INVENTION

The invention is in the field of diagnostics and therapeutics involving nucleic
5 acids.

BACKGROUND OF THE INVENTION

Nucleoside analogue reverse transcriptase inhibitors (NRTIs) represent the
cornerstone of antiretroviral therapy in HIV infection. Through their incorporation into
10 elongating viral DNA molecules transcribed by the HIV reverse transcriptase, they
effectively inhibit viral replication. However, NRTIs can also inhibit the human DNA
polymerase gamma (POL γ) (Martin *et al.*, 1994) and thereby mitochondrial DNA
(mtDNA) replication, leading to mtDNA depletion and drug toxicity (Brinkman *et al.*,
1998; Lewis and Dalakas, 1995; Kakuda, 2000). This mitochondrial toxicity (MT) leads to
15 a number of adverse effects including lactic acidosis, myopathy, cardiomyopathy,
neuropathy, liver steatosis, nephrotic toxicity and pancreatitis (Lewis and Dalakas, 1995;
others). The wide variety of clinical symptoms caused by NRTIs is reminiscent of the
complex array of symptoms produced by diseases resulting from mtDNA mutations (for
review see Wallace, 1999).

20 Early studies on zidovudine-induced myopathy have shown a decrease in total
mtDNA isolated from muscle biopsies in both humans (Arnaudo *et al.*, 1991) and rats
(Lewis *et al.*, 1992). In vitro studies with various anti-HIV nucleoside analogues have also
shown that NRTIs cause a reduction in the mitochondrial content of human
lymphoblastoid cells (Chen *et al.*, 1991; Zhang *et al.*, 1994), CEM cells (Medina *et al.*,
25 1994) and HepG2 cells (Pan-Zhou *et al.*, 2000). Recently, large hepatic mtDNA deletions
but no mtDNA depletion were reported in association with a fatal case of lactic acidosis
during antiretroviral therapy (Bartley *et al.*, 2001). It has been suggested that mtDNA
depletion (or deletion) may cause a decrease in mitochondrial RNA, mtDNA-encoded
protein synthesis and ultimately mitochondrial dysfunction (Lewis *et al.*, 1992). At the
30 cellular level, the consequences of such toxicity are decreased oxidative phosphorylation,
intracellular lipid accumulation and lactic acid accumulation. At the physiological level,
this may translate into hyperlactatemia that may or may not be accompanied by other

mitochondrial toxicity symptoms such as fatigue, rapid weight loss, lipid abnormalities, and liver steatosis. Chronic hyperlactatemia is likely a reflection of impaired hepatic lactate clearance (Brinkman, 2000) which may or may not find its etiology in the nucleoside analogue toxicity itself. Considering the long term nature of antiretroviral therapy, this recently identified syndrome of hyperlactatemia appears to be seen with increasing frequency in HIV infected patients on antiretroviral therapy (Lonergan et al., 2000; Gerard et al., 2000). Its presentation, severity and frequency are distinct from those of acute lactic acidosis, a rare NRTI adverse effect which is often fatal (Fortgang et al., 1995; Megarbane et al., 2000). However, whether hyperlactatemia is a risk factor for lactic acidosis remains unclear.

The diagnosis and treatment of patients with this NRTI-induced hyperlactatemia remains problematic. For example, it can be challenging to diagnose the condition because the early toxicity symptoms of fatigue and wasting are relatively common in AIDS patients and can resemble disease progression. Once mitochondrial toxicity is recognized, treatment may consist of terminating NRTI therapy and monitoring improvement in the patient condition and blood lactic acid levels (Brinkman, 2000; Moyle, 2000). Diagnosis of mitochondrial dysfunction may be made by muscle or liver biopsy, but this may not be practical for routine screening and monitoring. A random venous lactic acid (RVLA) measurement is a useful marker but its reliability is limited by its sensitivity to external factors that are difficult to control. The monitoring of RVLA in a cohort of antiretroviral-treated HIV positive patients has demonstrated that consecutive RVLA measurements were consistent within individuals and were frequently above the normal range (Harris et al., 2000). Moreover, a significant correlation has been found between abnormal RVLA and treatment with stavudine (D4T) and hydroxyurea, as well as length of time on D4T (Harris et al., 2000). However, elevated RVLA levels are not specific to nucleoside-related mitochondrial toxicity and can have other causes such as infection. There is little *in vivo* data available for nucleosides-related toxicities observed with NRTIs other than zidovudine.

30 SUMMARY OF THE INVENTION

In one aspect, the invention provides a method for monitoring toxicity of a drug treatment, comprising measuring the relative mitochondrial DNA content of cells in a subject undergoing treatment with the drug. The mitochondrial DNA content may be

measured relative to the amount of nuclear DNA in the cells of the subject. The amount of DNA may for example be measured by a polymerase chain reaction, such as a quantitative polymerase chain reaction, wherein amplification of the mitochondrial DNA is compared to amplification of a reference DNA. The methods of the invention may be used on human patients suffering from a disease, such as HIV infection, such as patients undergoing treatment with a nucleoside analogue (such as D4T). In alternative aspects, methods of the invention may be used to monitor the mitochondrial toxicity of test compounds in animal models, where for example the animal model subject is undergoing treatment with a drug. The assay may for example be conducted on cells extracted from a tissue, such as cells obtained from organ biopsies (which may for example be obtained post-mortem).

In one aspect, for example, the present invention discloses that mtDNA from peripheral blood mononuclear cells (PBMCs) is depleted in patients who are experiencing nucleoside-related mitochondrial toxicity (MT) symptoms. A semi-quantitative assay is accordingly provided to detect and monitor NRTI-related mitochondrial toxicity from a venous blood sample. In alternative embodiments, the methods of the invention may comprise the step of discontinuing treatment of the subject with a nucleotide analogue, such as D4T, when the relative mitochondrial DNA content of the cells falls below a predetermined level, such as when the predetermined level of mitochondrial DNA is 5, 10, 15, 20, 25, 30 or 35% of a baseline level of mitochondrial DNA, wherein the baseline level of mitochondrial DNA is measured before the subject is treated with the drug, or is measured in a control subject.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: PBMC mtDNA/nDNA ratios of A) HIV negative males, B) HIV positive/drug naïve males (no PI/NNRTI were detectable in plasma samples), C) HIV positive/symptomatic MT patients. The black bar represents the lowest mtDNA/nDNA ratio measured during antiretroviral therapy and the gray bar represents the highest ratio reached after interrupting the initial antiretroviral therapy.

Figure 2: Longitudinal analysis of venous lactate levels (left axis) and mtDNA levels (right axis) and antiretroviral drug regimen (bottom bar) over time, for the patients with MT symptoms. The bar is colored dark gray when the patients were on the drug regimen that led to MT, white when off all antiretroviral drugs, and hatched when receiving a new regimen that does not include D4T (see Table 1). This antiretroviral drug

data is based on the medical chart information, drug prescription dates and plasma drug levels. Pale gray regions indicate samples in which the plasma drug levels for PI and/or NNRTI were measured at >2 standard deviations below the average through concentration (according to the drug manufacturer's monograph). Note that for clarity and simplicity time is expressed as the distinct days on which the samples were collected.

Figure 3: Longitudinal analysis of mtDNA levels (left axis, expressed as the ratio mtDNA/nDNA) for patients receiving the antiretroviral regimen stavudine (d4T), didanosine (ddI) and efavirenz (EFV) over a time course shown in days (bottom axis). A) Two patients who did not have adverse effects. B) Three patients who did have adverse effects (hyperlactatemia, weight loss, +/- peripheral neuropathy). Open symbols = on therapy, close symbols = off therapy because of adverse side effects. Patients represented by squares also received hydroxyurea.

Figure 4: Typical LightCycler Real-Time PCR standard curves generated for the nuclear gene ASPOLG and the mitochondrial gene CCOI, using serial dilutions of the pooled DNA extracts from HIV negative male volunteers. The numbers (30 to 30,000) shown in the standard curve for the nuclear gene indicate the number of nuclear-genome equivalents included in each run. The same numbers were assigned in the standard curve for the mitochondrial gene (although they do not represent a calculated copy number of the mitochondrial gene). The nuclear-genome-equivalent content of the HIV negative DNA pool was determined by calibration with a control human DNA of known nuclear-genome-equivalent concentration (as for example may be available from Roche Applied Science, Laval, Quebec, Canada).

Figure 5: Comparative box plots of mtDNA/nDNA ratios between HIV uninfected males (mean \pm SD = 1.28 ± 0.38 , N=24), HIV infected asymptomatic/antiretroviral naive males (no detectable PI/NNRTI in plasma samples) (0.72 ± 0.19 , N=47), and HIV infected/antiretroviral treated symptomatic mitochondrial toxicity patients. For the latter, the -on therapy- (0.41 ± 0.08 , N=8) and -off therapy- (0.74 ± 0.13 , N=7) mtDNA/nDNA ratios are depicted. The lines indicate the maximum and minimum mtDNA/nDNA ratios observed within each group, the edges of the box indicate the 25% and 75% quartiles, the middle line indicates the median and the black square shows the mean mtDNA/nDNA ratio.

DETAILED DESCRIPTION OF THE INVENTION

In one aspect, the invention provides an assay to quantify mitochondrial DNA (mtDNA) in peripheral blood cells and thereby determine whether the mtDNA levels are at levels indicative of mitochondrial deficit, such as may be caused by toxicity of a therapeutic treatment. The invention provides assays to determine the relative amount of mitochondrial DNA in a subject, such as a subject undergoing drug treatment. The subject may for example be a human patient undergoing treatment for an HIV infection with a nucleic acid precursor such as a nucleoside or nucleotide analogue. The assays of the invention may include PCR assays, such semi-quantitative or quantitative PCR involving the co-amplification of a mitochondrial sequence and a reference sequence, such as a genomic sequence. Information from such assays may be evaluated to provide a ratio of mitochondrial DNA to nuclear DNA in the cells of the subject.

For example, such assays may be carried out in HIV patients on antiretroviral therapy. In one aspect of the invention, antiretroviral therapies may therefore be modulated in accordance with the results of the mtDNA assays of the invention. In alternative aspects of the invention, sample may be tested from patients undergoing therapy with nucleic acid precursors, such as nucleoside or nucleotide analogues. Nucleoside analogs may for example include AZT and ZDV (Retrovir), ddI (Videx and Videx EC) ddC (Hivid) d4T (Zerit) 3TC (Epivir) ABC (Ziagen). Nucleoside analogues are any modified versions of a natural nucleoside. Nucleoside analogs may take the place of the natural nucleosides, blocking the completion of a viral DNA chain during infection of a new cell by HIV. Alternative nucleic acid precursors include nucleotide analogues, such as Cidofovir (also known as HPMPC). Nucleoside analogs and other nucleic acid precursors may also be used in cancer chemotherapy, to inhibit replication of cancer cells.

Samples from patients that are HIV positive may be tested in various aspects of the invention, including such patients who are undergoing nucleoside analogue therapy. Similarly, cancer patients may be monitored with diagnostic assays of the invention, including cancer patients undergoing therapy with a nucleic acid precursor.

In the examples illustrating various aspects of the invention, mitochondrial DNA is shown to be depleted in peripheral blood cells from patients undergoing therapy with a nucleic acid precursor who are suffering from antiretroviral drug-related hyperlactatemia and other mitochondrial toxicity symptoms such as fatigue and rapid weight loss. This depletion preceded a rise in venous lactic acid levels, an observation that is consistent with

hyperlactatemia being a consequence of mtDNA depletion. In some embodiments, assays of the invention could therefore provide clinical information before mitochondrial toxicity develops or becomes severe enough to be accompanied by hyperlactatemia. In some cases, even short periods of time with reduced plasma drug concentration are shown to result in increased mtDNA/nDNA ratios, showing that the tests of the invention may advantageously be performed while patients are actively undergoing therapy.

The depletion in mtDNA levels is shown to be reversible in some patients, as shown by the examples herein which illustrate a rise in mtDNA/nDNA ratios following antiretroviral therapy interruption. In the relevant examples, this was accompanied by a gradual return to normal VLA levels. It is shown in the examples that mtDNA levels were significantly lower in the HIV positive control group than in the HIV negative one, a difference that was not explained by the lower CD4 counts in the former. Accordingly, in one aspect the invention provides a diagnostic test that may provide information indicative of HIV infection. It was also found that within the HIV positive control group, storage prior to DNA extraction showed no significant correlation with the mtDNA/nDNA ratios measured (data not shown). In some cases, it was found that severe symptoms may occur when the mtDNA levels fall below approximately 30% to 20% of normal. Alternative measures of mtDNA depletion that may suggest clinical intervention are mtDNA to nDNA ratios of less than 0.5, 0.45, 0.4, 0.35 or 0.3.

In alternative embodiments, drug treatment may be discontinued when the ratio of mtDNA to nDNA, as determined herein, falls below a threshold value such as 0.5, 0.45, 0.4, 0.35 or 0.3 as measured with respect to a control sample. Methods of the invention may comprise treating the patient with an alternative nucleoside analogue after discontinuing treatment of the subject with a particular nucleoside such as D4T. Alternatively, such patients may be treated with mitochondrial therapeutics, i.e. compositions of benefit to mitochondria, such as mitochondrial enzyme co-factors or precursors. In some embodiments, such mitochondrial therapeutics may for example be selected from the group consisting of riboflavin (vitamin B2), coenzyme Q10, vitamin B1 (thiamine), vitamin B12, vitamin K, l-acetyl carnitine, N-acetyl cysteine and nicotinamide.

In alternative embodiments, the rate of change of mtDNA concentration over a time period may be determined to provide additional diagnostic information. For some patients, a relatively rapid decrease in the relative amount of mtDNA may be indicative of drug toxicity or a disease state. For example, as shown in Figure 3B, a relatively rapid

decrease of on the order of 50% or more (or more than 40% in some cases) in the relative amount of mtDNA compared to nDNA over a period of less than eight to ten days may be indicative that a patient will eventually have adverse effects from a drug, and may therefore need to be monitored more closely, and may eventually need to be rotated to
5 alternative treatment or have drug treatment discontinued.

As was the case for other studies of hyperlactatemia (Lonergan et al., 2000; Gerard et al., 2000; John et al., 2001), all the patients were receiving D4T as part of their drug regimen at the time the toxicity developed. It was surprisingly discovered that the mtDNA/nDNA ratios measured while off antiretroviral therapy were very similar to those
10 observed once patients resumed nucleoside-containing therapy that excluded D4T. (Table 1, Figure 2 patients 3, 4, 5). Accordingly, one aspect of the invention provides for discontinuance of a drug therapy when a threshold depletion of mtDNA is detected, in conjunction with a switch to an alternative drug. For example, patients may switch from one nucleoside analogue regimen to an alternative nucleoside analogue regimen.

15 The frequency of lactic acidosis has been estimated in a 1995 retrospective study to lie between one and two cases per 1000 person-year treated with NRTIs (Fortgang et al., 1995). However, another study with a broadened case definition of hyperlactatemia accompanied by either abdominal symptoms or unaccounted for elevated alanine transferase estimated the incidence at 20.9 cases per 1000 person-years of treatment with
20 NRTIs (Lonergan et al., 2000). A large proportion of patients receiving antiretroviral therapy in our cohort exhibit mild to moderate chronic hyperlactatemia, most of them asymptomatic. The assay of the invention may be used to monitor and evaluate the clinical consequences of mitochondrial toxicity and chronic hyperlactatemia in such patients.

25 In one aspect of the invention, protocols are provided that avoid the necessity to determine mtDNA copy number *per se*, facilitating instead a determination of the relative amount of mtDNA, such as the amount relative to a nDNA sequence. In some aspects, this approach may simplify the diagnostic assays of the invention. For example, as shown in Figure 4, numbers (30 to 30,000) representing nuclear-genome-equivalents are assigned to
30 nDNA amplification standards, as determined by calibration with a control human DNA of known nuclear-genome-equivalent concentration. The same numbers are arbitrarily assigned to the corresponding standard curves for the mitochondrial gene (although they do not represent a calculated copy number of the mitochondrial gene). In an alternative

approach, the numbers representing nuclear-genome-equivalents may be arbitrarily assigned to the nDNA amplification standards, based only on the degree of sample dilution (so that the number reflect the relative copy number of nuclear-genome-equivalents, but not the absolute value of such equivalents), and these arbitrary numbers
5 may similarly be assigned to the mtDNA amplification standards. The results of the assays of the invention may then be expressed by the ratio of mtDNA to nDNA, without the need to determine absolute mtDNA copy numbers. In such embodiments, it may be preferable to utilize an initial concentration of sample DNA that provides sufficient PCR template so that the number of amplification cycles is within the range which provides the most
10 reliable results, such as from a minimum of any integer from 5 to 15 up to a maximum of any integer from 15 to 40.

A process for comparing the relative abundance of NA sequences, comprising:

- a) measuring the amplification kinetics of a nuclear NA sequence under a nuclear amplification reaction condition in a first nuclear control sample and in a second nuclear control sample, to obtain control nuclear
15 amplification measurements, wherein the first and the second nuclear control samples have different concentrations of the nuclear NA sequence;
- b) constructing a control nuclear NA sequence dataset from the control nuclear amplification measurements, to obtain a model standard
20 relationship between amplification kinetics and concentration for the nuclear NA sequence;
- c) measuring the amplification kinetics of a mitochondrial NA sequence under a mitochondrial amplification reaction condition in a first mitochondrial control sample and in a second mitochondrial control
25 sample, to obtain control mitochondrial amplification measurements, wherein the first and the second mitochondrial control samples have different concentrations of the mitochondrial NA sequence;
- d) constructing a control mitochondrial NA sequence dataset from the control mitochondrial amplification measurements, to obtain a model
30 standard relationship between amplification kinetics and concentration for the mitochondrial NA sequence;

e) measuring the amplification kinetics of the nuclear NA sequence under the nuclear amplification reaction conditions in a test sample, to obtain a test sample nuclear amplification measurement;

5 f) applying the model standard relationship between amplification kinetics and concentration for the nuclear NA sequence to the test sample nuclear amplification measurement, to obtain a test sample nuclear NA sequence concentration measurement;

10 g) measure the amplification kinetics of the mitochondrial NA sequence under the mitochondrial amplification reaction conditions in the test sample, to obtain a test sample mitochondrial amplification measurement;

15 h) applying the model standard relationship between amplification kinetics and concentration for the mitochondrial NA sequence to the test sample mitochondrial amplification measurement, to obtain a test sample mitochondrial NA sequence concentration measurement;

i) comparing the test sample nuclear NA sequence concentration measurement to the test sample mitochondrial NA sequence concentration measurement, to determine the relative concentration of the mitochondrial NA sequence compared to the nuclear NA sequence in the test sample.

20 In alternative aspects of the invention, cells for use in assays of the invention may be obtained, for example by biopsy, from a variety of tissues, such as from heart, brain, kidney, fat or liver.

In alternative embodiments, the diagnostic tests of the invention may be used for the diagnosis of a disease condition. For example, measuring the relative amount of a
25 mitochondrial DNA in cells in a sample from a subject, such as a sample of peripheral blood, may provide information relating to diseases or symptoms such as male infertility, organ failure, hepatitis A, B or C infection, HIV infection, arthritis, a neurological disease (including but not limited to Alzheimer's, Parkinson's, Huntington's). The diagnosis of such conditions may for example be undertaken when the conditions are treated with a drug,
30 such as a nucleic acid precursor, or the conditions are caused by such a drug.

In alternative aspects, the invention provides kits having components for use in methods of the invention. Such kits may comprise PCR components, as set out in detail below, including PCR primers specific for a mtDNA sequence and for a nDNA sequence.

Such kits may also include written instructions for carrying out the methods of the invention as described herein.

In alternative embodiments, a variety of techniques may be used to measure the relative amount of a mitochondrial DNA in cells. Methods of quantitative PCR are for example disclosed in the following documents, all of which are incorporated herein by reference: United States Patent No. 6,180,349 issued to Ginzinger, *et al.* January 30, 2001; United States Patent No. 6,033,854 issued to Kurnit, *et al.* March 7, 2000; and United States Patent No. 5,972,602 issued to Hyland, *et al.* October 26, 1999.

10 Example 1

As illustrated in the following examples, nucleoside-related mitochondrial toxicity is associated with a significant decrease in blood cell mtDNA content, an effect that is reversible upon therapy interruption. An assay is provided to monitor mitochondrial toxicity, for example in patients on antiretroviral therapy. Methods of the invention may be adapted to assess the toxicity of other drugs and to monitor the mitochondrial health of patients with inherited diseases that affect mtDNA levels.

Materials and Methods

Longitudinal blood samples were studied retrospectively from 8 patients whose antiretroviral therapy was interrupted because of mitochondrial toxicity symptoms. Their symptoms included moderate hyperlactatemia, fatigue, rapid weight loss and low anaerobic threshold in cardiopulmonary testing. Total DNA was extracted from blood cells and both a nuclear gene and a mitochondrial gene were amplified and quantified by Real-Time PCR using hybridization probes. The mtDNA levels were expressed as a ratio of the mitochondrial over nuclear DNA (mtDNA/nDNA).

25 Sample collection and DNA extraction

Buffycos were collected from the same blood samples used for plasma viral load determination and stored frozen at -70°C until used. Plasma viral loads were measured using the Amplicor Ultra-Sensitive HIV-1 Monitor assay (Roche Molecular Diagnostic Systems, Branchburg, New Jersey). Total DNA was extracted from 200 μL of buffycos using the QIAamp DNA Blood Mini kit (QIAGEN, Mississauga, Ontario, Canada) according to the manufacturer's protocol, and resuspended in 200 μL of elution buffer. For the standard curves, similar samples were collected from 24 HIV negative male

volunteers and the DNA was extracted and pooled. The nuclear genome equivalent (g.eq.) content of the HIV negative DNA pool was determined by calibration with control kit human DNA of known nuclear g.eq. concentration (Roche Molecular Biochemicals, Laval, Quebec, Canada).

5 **Random venous lactic acid measurement**

Venous specimens for lactic acid determination were collected in sodium fluoride/potassium oxalate tubes, with normal tourniquet and no specific patient instruction other than the avoidance of fist clenching or hand pumping. The laboratory reference range is 0.7 to 2.1 mmol/L.

10 **Quantitative real-time PCR**

For the mtDNA CCOI gene, the CCOI1F 5'-TTCGCCGACCGTTGACTATT-3' and CCOI2R 5'-AAGATTATTACAAATGCATGGGC-3' primers were used for the PCR amplification and the oligonucleotides 3'-Fluorescein-labeled CCOIPR1 5'-GCCAGCCAGGCAACCTTCTAGG-F-3' and 5'LC Red640-labeled CCOIPR2 5'-L-AACGACCACATCTACAACGTTATCGTCAC-P-3', the 3' end of the latter blocked with a phosphate molecule, were used as hybridization probes. For the nDNA ASPOL γ gene, the ASPG3F 5'-GAGCTGTTGACGGAAAGGAG-3' and ASPG4R 5'-CAGAAGAGAATCCCGGCTAAG-3' primers were used for the PCR and the oligonucleotides 3'-Fluorescein-labeled ASPGPR1 5'-GAGGCGCTGTTAGAGATCTGTCAGAGA-F-3' and 5'LC Red640-labeled, 3'-Phosphate-blocked ASPGPR2 5'-L-GGCATTTCCTAAGTGAAGCAAGCA-P-3' were used as hybridization probes. The real-time PCR reactions were done separately and in duplicate for each gene, using the LightCycler FastStart DNA Master Hybridization Probes kit (Roche Molecular Biochemicals, Laval, Quebec, Canada). The PCR reactions contained 5 mM MgCl₂, 0.5 μ M of each primer, 0.1 μ M 3'-Fluorescein probe, 0.2 μ M 5'LC Red640 probe and 4 uL of a 1:10 dilution of the DNA extract in elution buffer. The PCR amplification consisted of a single denaturation/enzyme activation step of 10 min at 95°C followed by 45 cycles of 0 s/95°C, 10 s/60°C, 5 s/72°C, with a 20°C/s temperature transition rate. The gain settings were F1=1, F2=8 and a single fluorescence acquisition was made at the end of each annealing step. An external standard curve of 30, 300, 3000, and 30000 nuclear g.eq. was included in each LightCycler run, and the same nuclear g. eq values were used for both the nuclear (ASPOL γ) and the mitochondrial (CCOI) genes.

The data were analyzed using the second derivative maximum of each amplification reaction and relating it to its respective standard curve. Results from the quantitative PCR were expressed as the relative ratio of the mean mtDNA g.eq. of duplicate measurements over the mean nDNA g.eq. of duplicate measurements for a given extract
5 (mtDNA/nDNA), a ratio arbitrarily set around 1.0 by the fact that the same nuclear g. eq. values were used to generate both standard curves.

In some embodiments, PCR methods of the invention may be real-time polymerase chain reactions wherein an amplification product is detected with a hybridization probe, such as described above using the LightCycler FastStart DNA Master Hybridization
10 Probes kit (Roche Molecular Biochemicals, Laval, Quebec, Canada) or alternative commercially available techniques such as ABI Taqman® technology (using for example an ABI Prism 7700 instrument to detect accumulation of PCR products continuously during the PCR process, Applied Biosystems, Foster City, California, U.S.A.). Alternative PCR methods and variations on the forgoing methods may be adopted, as for example are
15 disclosed in the following U.S. Patents which are hereby incorporated by reference:
6,180,349 (Ginzinger et al; Jan. 30, 2001); 6,033,854 (Kuit et al; March 7, 2000);
5,972,602 (Hyland; Oct. 26, 1999); 5,476,774 and 5,219,727 (Wang; Dec. 19, 1995 and
June 15, 1993); 6,174,670 (Wittwer et al; Jan. 16, 2001); 6,143,496 (Brown; Nov. 7,
2000); 6,090,556 (Kato; July 18, 2000); 6,063,568 (Gerdes et al; May 16, 2000).

20

Plasma drug levels

The concentration of protease inhibitors (PIs) (indinavir, ritonavir, saquinavir, nelfinavir and lopinavir) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) (nevirapine, delavirdine and efavirenz) were determined in the stored plasma samples that were collected for viral load testing. This was done using high performance liquid chromatography (HPLC) (HP 1100, Agilent Palo Alto, CA) coupled with tandem mass spectrometry (API-2000 LC/MS/MS System, AB/MDS-Sciex, Foster City, CA). Briefly, the PIs and NNRTIs were extracted with acetonitrile and precipitated plasma proteins were separated by filtration with Ultrafree-MC Filters (Millipore, Bedford, MA). The drugs in the filtrate were partially separated by HPLC on a Zorbax XDB C-18 column (Agilent Palo Alto, CA) and quantified by standard methods on the mass spectrometer. The samples were collected in acid citrate dextrose (ACD) tubes that dilute the blood somewhat and the time at which the last dose was administered was unknown. For these reasons, the plasma drug levels were considered to be a qualitative evaluation of whether the antiretrovirals were taken regularly and reaching the blood circulation.

Statistical analysis

The Wilcoxon signed-rank test was used to assess paired differences between measurements (Table 3). Non-parametric Spearman's rho correlation coefficient was used to assess the correlation between clinical tests and the mtDNA/nDNA ratios.

20

Results

The mitochondrial toxicity symptoms (N=8) were associated with markedly low mtDNA/nDNA ratios, 70% lower than HIV negative controls (N=24) and 45% lower than HIV positive/antiretroviral naive controls (N=47). The mtDNA ratios increased significantly following discontinuation of therapy (p=0.016). The decline in mtDNA preceded the increase in venous lactic acid levels and similarly, the post-therapy rebound in mtDNA appeared to precede a return to normal lactate levels. No significant correlation was observed between CD4 count (p=0.170) or platelet count (p=0.141) and the mtDNA/nDNA ratios.

10 Patient characteristics

We retrospectively studied 8 HIV infected individuals enrolled in the Drug Treatment Program at the B.C. Centre for Excellence in HIV/AIDS at St.Paul's Hospital (Table 1). The patients experienced mitochondrial toxicity (MT) symptoms that included chronic hyperlactatemia, fatigue, rapid weight loss and low anaerobic threshold during cardiopulmonary exercise testing (data not shown). Two of the patients were on a drug regimen that consisted of 4 nucleoside analogues, 2 were taking hydroxyurea and all were receiving D4T at the time their MT symptoms developed. As a result of this drug toxicity, all had their antiretroviral therapy interrupted by their treating physician and their lactate levels monitored by RVLA over time. At the time of therapy interruption, 7/8 had a plasma viral load that was below the limit of detection of 50 copies/mL of plasma. After stopping antiretroviral therapy, MT symptoms gradually disappeared in all patients. The mean time off therapy was 15.6 weeks and 5/8 patients subsequently resumed antiretroviral therapy with a different drug regimen that excluded D4T and DDI, and achieved an undetectable plasma viral load. Two of the patients (4 and 5) had elevated liver enzymes prior to initiating their pre-interruption regimen. All others showed normal liver enzymes, INR and albumin levels before developing MT symptoms (data not shown).

25 MtDNA /nDNA ratios and antiretroviral therapy

Longitudinal blood samples from 8 patients (between 6 and 17 distinct samples per patient, covering a period of 22 to 28 months) were collected before, during and after the antiretroviral therapy interruption and their mtDNA/nDNA ratios were determined (Figure 1c, 2; Table 2). As a control, mtDNA/nDNA ratios were determined for 24 healthy HIV negative males and 47 HIV positive drug naive males (Figure 1a,b; Table 2).

A statistical comparison of the ratios obtained with the various groups is presented in Table 3. The mean mtDNA/nDNA ratio of the HIV negative controls was significantly higher than that of the HIV positive/drug naive group. In the calculation of the pre-therapy interruption mean, all samples were considered, including those for which prescribed PI and NNRTI plasma drug levels were either undetectable or measured at >2 standard deviations below the average trough concentration (according to the drug manufacturer monograph) (Figure 2). Post-therapy interruption samples included all samples collected after therapy interruption with no limitations on time. Patient 1 was excluded from this analysis since there was no buffycoat available from the period off antiretrovirals. Five of the 8 patients eventually resumed antiretroviral therapy that excluded the nucleoside analogues D4T and DDI from the new regimens (Table 1). All samples collected during that time were included in the calculation of the off D4T mean ratio.

For the 8 MT patients together, the mean mtDNA/nDNA ratio observed before therapy interruption (but at least one month after initiation of their last drug regimen) was significantly lower than those obtained for either the HIV negative or the HIV positive/antiretroviral naive control groups ($p < 0.001$). Both the mean ratios measured during complete therapy interruption and off D4T therapy (which include off all antiretrovirals as well as on antiretroviral regimen that excludes D4T) were very similar to the mean obtained for the HIV positive/drug naive controls. In fact, the mean mtDNA/nDNA ratio of all the samples collected pre-therapy interruption was significantly lower than both the mean ratio off all antiretrovirals ($p = 0.016$) and the mean ratio off D4T ($p = 0.008$) (Table 3).

Several additional patient clinical test results were investigated to determine whether they showed a relationship with the mtDNA/nDNA ratio. No significant correlation between the mtDNA/nDNA ratio and CD4 count, both in the HIV positive/antiretroviral naive group ($p = 0.593$) and in the MT patient group ($p = 0.170$). Platelets contain a few mtDNA molecules per cell (Shuster et al., 1988) which may influence the mtDNA/nDNA ratio. Platelet data was not available for the control groups but for the MT group, there was no significant correlation between the ratio and platelet count ($p = 0.14$). Similarly, for the MT patients, no correlation was found between the ratio and the white blood cell count ($p = 0.21$), the alanine aminotransferase (ALT) ($p = 0.47$), or the INR. However, a weak correlation was found between the mtDNA/nDNA ratio and the AST ($p = 0.02$) as well as the albumin level ($p = 0.02$).

MtDNA and lactate levels

In patients 1, 4 and 8 (Figure 2) the mtDNA depletion clearly preceded the hyperlactatemia (earlier lactate data was unavailable for the other five patients). Similarly, in patients 4, 6 and 8, the time required for mtDNA levels to rebound was similar or shorter than that needed for the hyperlactatemia to normalize (0.5-2.1 mmol/L range). In several instances the mtDNA rebound preceded plasma viral load rebound (data not shown). Based on the limited mtDNA/nDNA data available, the maximum mtDNA half-life was estimated to range from 4.5 weeks (patient 3) to 8 weeks (patient 4) and the maximum mtDNA doubling times were estimated to range from 4 (patient 5) to 16 weeks (patient 1). Short lapses in therapy (as seen for patients 3, 4, 7) as well as extremely low circulating drug levels (patients 4, 6, 7) also affected mtDNA levels upward. Based on the data available, the maximum time off drugs before lactate levels returned to the normal range varied from 4 weeks (patient 8) to 28 weeks (patient 5). Furthermore, lactate levels remained within the normal range for months after resuming therapies that included 3TC + abacavir (ABA) or zidovudine (AZT). The difference between venous lactate levels on and off antiretroviral therapy did not reach significance (Table 3). This is most likely due to the lag time between changes in therapy and changes in lactate levels, a lag that was also present between changes in mtDNA/nDNA ratios and lactate levels (see Figure 2, patients 1 and 4).

Table 1. Characteristics of the eight patients with symptomatic mitochondrial toxicity and their antiretroviral therapy regimens.

patient	Age/ Gender	Drug Regimen ^c	Before stopping therapy			Off therapy			After resuming therapy	
			Time on D4T ^a (weeks)	last HIV-1 pVL ^b (copies/mL)	Time off Therapy (weeks)	highest HIV-1 pVL (copies/mL)	Drug Regimen	Time to HIV-1 pVL <50 copies/mL (weeks)		
1	47/M	D4T/DDI/3TC/ABA/HU	175	<50	13	223,000	SAQ/RIT/NEV	12		
2	41/M	D4T/DDI/3TC/SAQ/DEL/NEL/NEV/ABA/HU	144	<50	45+	178,000	N/A	N/A		
3	44/M	D4T/DDI/3TC/ABA	59	90	15	177,000	3TC/ABA/NEV/ABR	18		
4 ^d	48/M	D4T/3TC/SAQ/RIT	58	<50	17	584,000	3TC/ABA/NEV/ABR	20		
5	41/M	D4T/DDI/EFV	33	<50	17	425,000	3TC/SAQ/RIT/EFV	17		
6	57/M	D4T/DDI/EFV	33	<50	17	750,010	AZT/3TC/SAQ/RIT/EFV	17		
7	44/M	D4T/DDI/3TC/SAQ/IND/NEV/ABA/ABR	192	<50	28+	63,300	N/A	N/A		
8	43/M	D4T/IND/DEL	143	<50	26+	138,000	N/A	N/A		

- a) Time the patient had continuously been prescribed D4T as part of their drug regimen, before stopping therapy.
- b) Last plasma viral load before stopping antiretrovirals.
- c) The abbreviations used are: D4T, stavudine; DDI, didanosine; 3TC, lamivudine; ABA, abacavir; SAQ, saquinavir; IND, indinavir; RIT, ritonavir; NEL, nelfinavir; ABR, lopinavir; DEL, delavirdine; NEL, nelfinavir; EFV, efavirenz; HU, hydroxyurea.
- d) Patient co-infected with hepatitis C virus.

e) Table 2. Mitochondrial DNA/nuclear DNA ratios measured for the different groups.

HIV status/Antiretrovirals status	N ^a (number of mtDNA/nDNA)	mtDNA/nDNA	
		mean \pm S.D.	(range)
HIV negative	24 (24)	1.283 \pm 0.377	(0.766-2.441)
HIV positive/Antiretroviral naïve	47 (47)	0.717 \pm 0.189	(0.368-1.098)
HIV positive with MT/on therapy	8 (37)	0.392 \pm 0.143	(0.184-0.856)
HIV positive with MT/off therapy	8 (17)	0.712 \pm 0.203	(0.394-1.228)
HIV positive with MT/off D4T	8 (38)	0.698 \pm 0.171	(0.394-1.228)

a) N = number of individuals within a given group, number of mtDNA/nDNA refers to the number of individual data points considered in the calculation of the mean value for that group.

On therapy data are those gathered while the patients were on their last regimen since \geq 1 month.

Off therapy means off all antiretrovirals

Off D4T means off all antiretrovirals and/or on ARV therapy that does not include D4T.

Table 3.

Comparison of mean values	data compared	P value ^a
HIV negative vs 8 patients with MT/on ARV	mtDNA/nDNA ratio	<0.001
HIV negative vs HIV positive/ARV naïve	mtDNA/nDNA ratio	<0.001
HIV positive/ARV naïve vs 8 patients with MT/on ARV	mtDNA/nDNA ratio	<0.001
8 patients with MT/on ARV vs off all ARV	mtDNA/nDNA ratio	0.016
8 patients with MT/on ARV vs off D4T	mtDNA/nDNA ratio	0.008
8 patients with MT/on ARV vs off all ARV	lactate	0.313
8 patients with MT/on ARV vs off D4T	lactate	0.109

a) Determined by Wilcoxon signed-rank test.

Example 2

In one aspect, it has been found that assays of the invention may be used on post-mortem tissues to provide information relating to organ failure characterized by

mitochondrial damage. In this example, post mortem analysis of tissues correlated well with cause of death. In a case where the cause of death was lactic acidosis and hepatic steatosis, mtDNA/nDNA ratios were reduced in liver compared to HIV+ and HIV- control samples. In a case where kidney failure was present at death, mtDNA/nDNA ratios were
5 significantly reduced in kidney tissues compared to controls.

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25

Conclusion

Although various embodiments of the invention are disclosed herein, many adaptations and modifications may be made within the scope of the invention in accordance with the common general knowledge of those skilled in this art. Such
30 modifications include the substitution of known equivalents for any aspect of the invention in order to achieve the same result in substantially the same way. Numeric ranges are inclusive of the numbers defining the range. In the specification, the word "comprising" is used as an open-ended term, substantially equivalent to the phrase "including, but not

limited to", and the word "comprises" has a corresponding meaning. Citation of references herein shall not be construed as an admission that such references are prior art to the present invention. All publications, including but not limited to patents and patent applications, cited in this specification are incorporated herein by reference as if each
5 individual publication were specifically and individually indicated to be incorporated by reference herein and as though fully set forth herein. The invention includes all embodiments and variations substantially as hereinbefore described and with reference to the examples and drawings.

WHAT IS CLAIMED IS:

1. A method of monitoring toxicity of a drug treatment, comprising measuring the
5 relative amount of a mitochondrial DNA in cells in a sample from a subject
undergoing treatment with the drug, wherein the sample is a peripheral blood
sample and the drug is a nucleic acid precursor.
2. The method of claim 1, wherein the mitochondrial DNA content is measured
10 relative to the amount of a nuclear DNA in the cells of the subject.
3. The method of claim 1 or 2, wherein the amount of DNA is measured by a
polymerase chain reaction.
- 15 4. The method of claim 3 wherein the polymerase chain reaction is a quantitative
polymerase chain reaction, wherein amplification of the mitochondrial DNA is
compared to amplification of a reference DNA.
5. The method of any one of claims 1 through 4 wherein the subject is a human
20 patient suffering from an HIV infection.
6. The method of any one of claims 1 through 4 wherein the subject is a human
patient suffering from cancer.
- 25 7. The method of any one of claims 1 through 6 wherein the drug is a nucleoside or
nucleotide analogue.
8. The method of claim 7, wherein the nucleoside analogue is selected from the group
consisting of AZT, ddI, ddC, d4T, 3Tc, Abacavir, Tenofovir and D4T.
30
9. The method of claim 7, wherein the nucleoside analogue is D4T.

10. The method of claim 8 or 9, further comprising the step of discontinuing treatment of the subject with the nucleoside analogue when the relative mitochondrial DNA content of the cells falls below a predetermined level.
- 5 11. The method of claim 8 or 9, further comprising the step of treating the patient with an alternative nucleoside analogue after discontinuing treatment of the subject with the nucleoside analogue.
- 10 12. The method of claim 8 or 9, wherein the predetermined level of mitochondrial DNA is expressed as a ratio of mtDNA to nDNA with reference to a standard mtDNA/nDNA ratio set at 1, and wherein the predetermined level is a ratio of 0.45 or less.
13. The method of claim 1, wherein the subject is a non-human animal.
- 15 14. The method of claim 3, wherein the polymerase chain reaction is a real-time polymerase chain reaction wherein an amplification product is detected with a hybridization probe.
- 20 15. The method of any one of claims 1 through 5 wherein the drug is a reverse transcriptase inhibitor.
16. The method of any one of claims 1 through 4 wherein the subject is a human patient suffering from an hepatitis A, B or C infection.
- 25 17. The method of any one of claims 1 through 4 wherein the subject is a human patient suffering from arthritis infection.
18. The method of any one of claims 1 through 4 wherein the subject is a human patient suffering from a neurological disease.
- 30 19. The method of claim 18, wherein the neurological disease is Alzheimer's disease, Parkinson's disease or Huntingtin's disease.

20. A method of diagnosis of a disease condition, comprising measuring the relative amount of a mitochondrial DNA in cells in a sample from a subject, wherein the sample is a peripheral blood sample and the disease condition is selected from the group consisting of male infertility and organ failure.
- 5

Figure 1

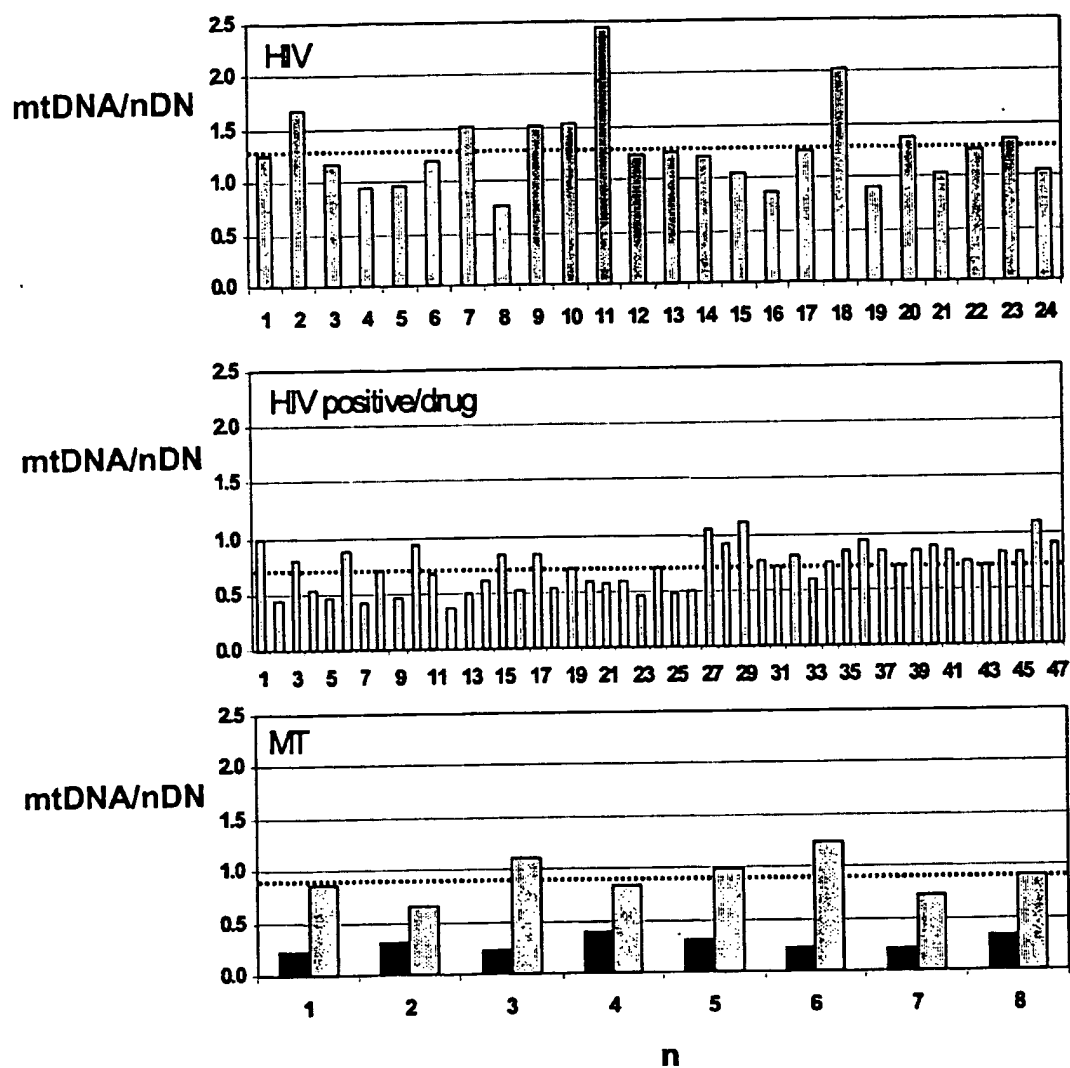


Figure 2

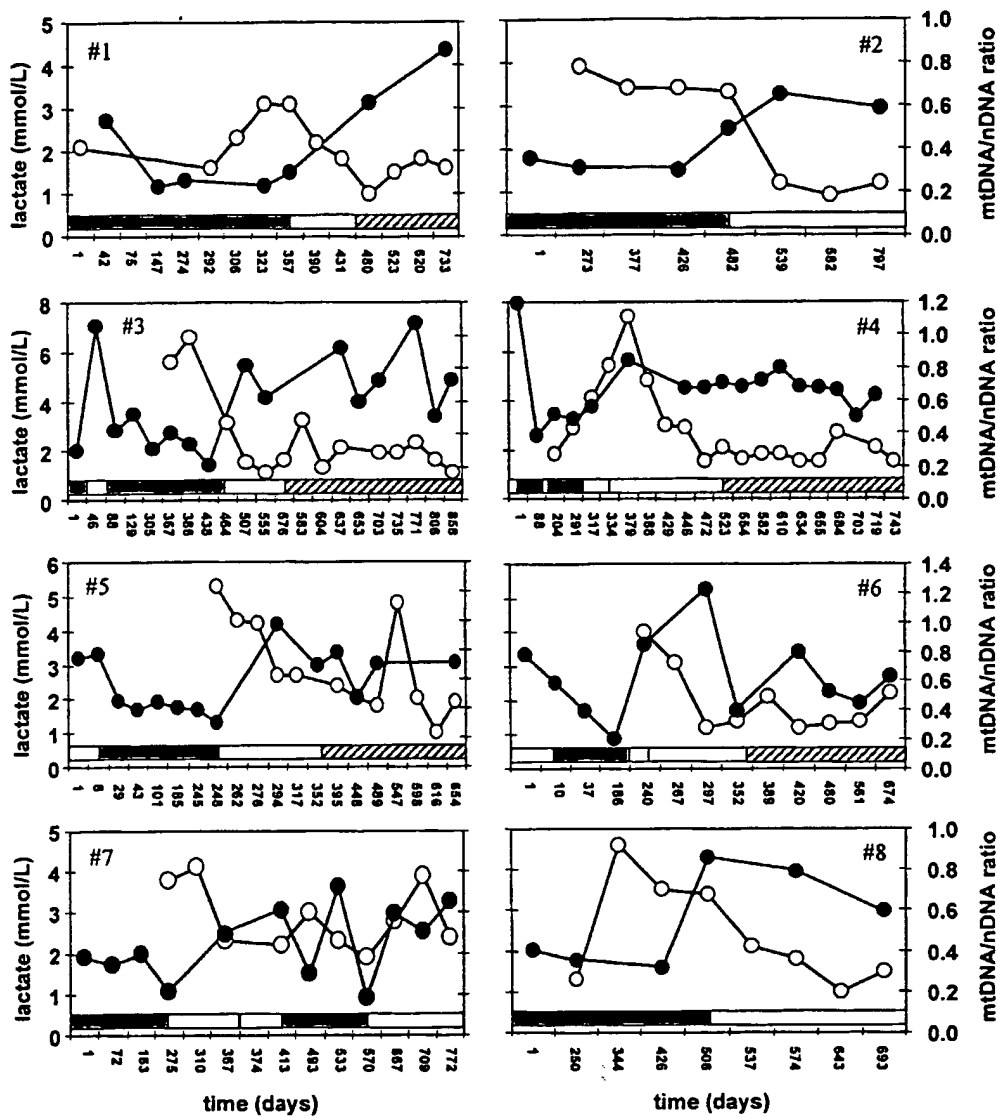


Figure 3

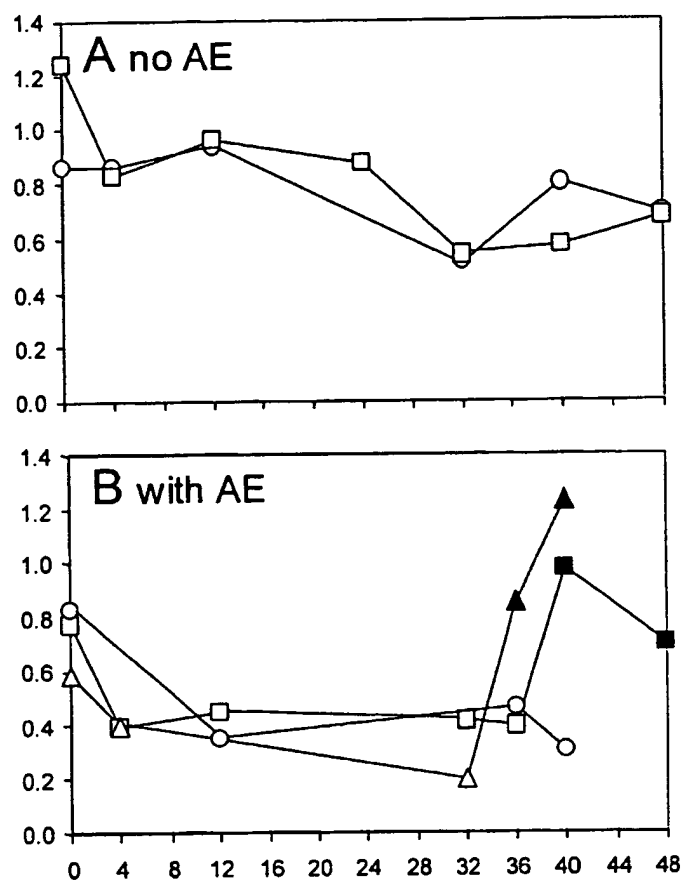


Figure 4

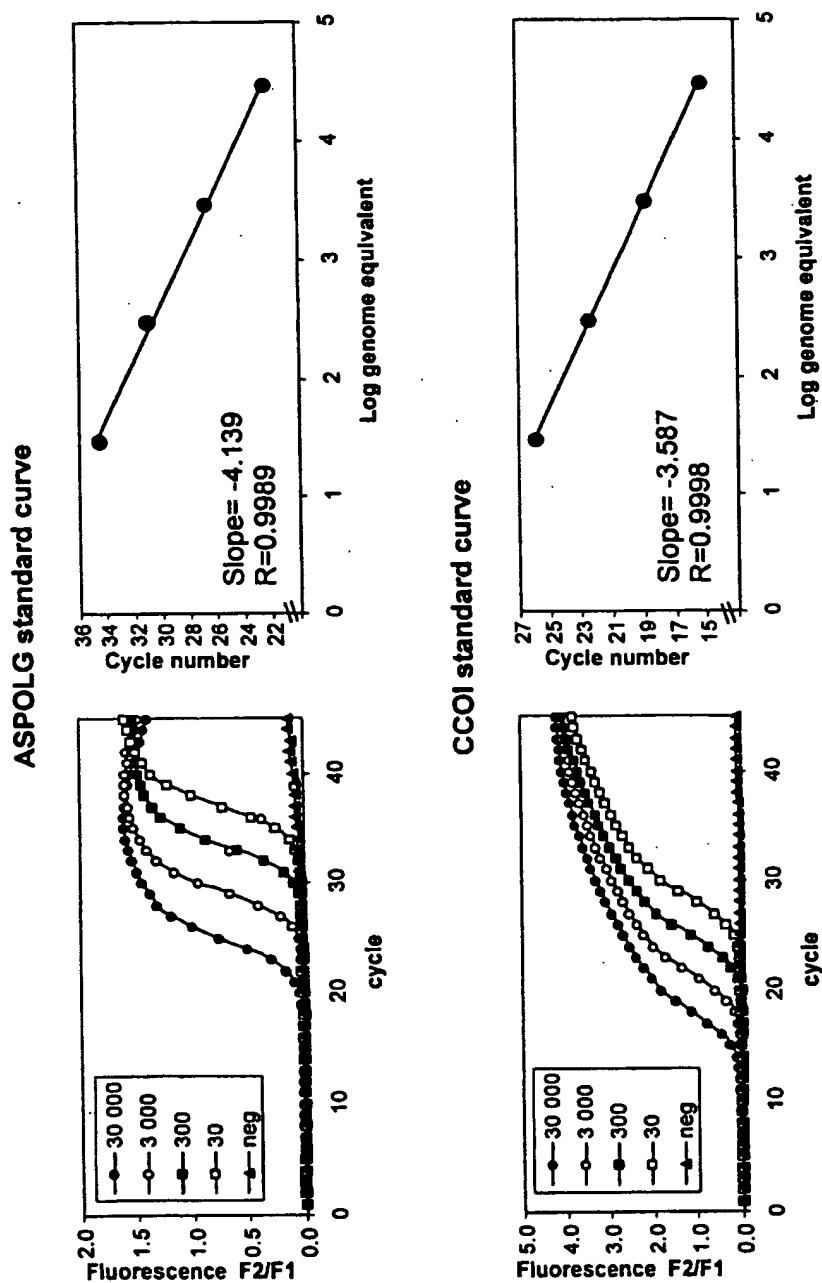
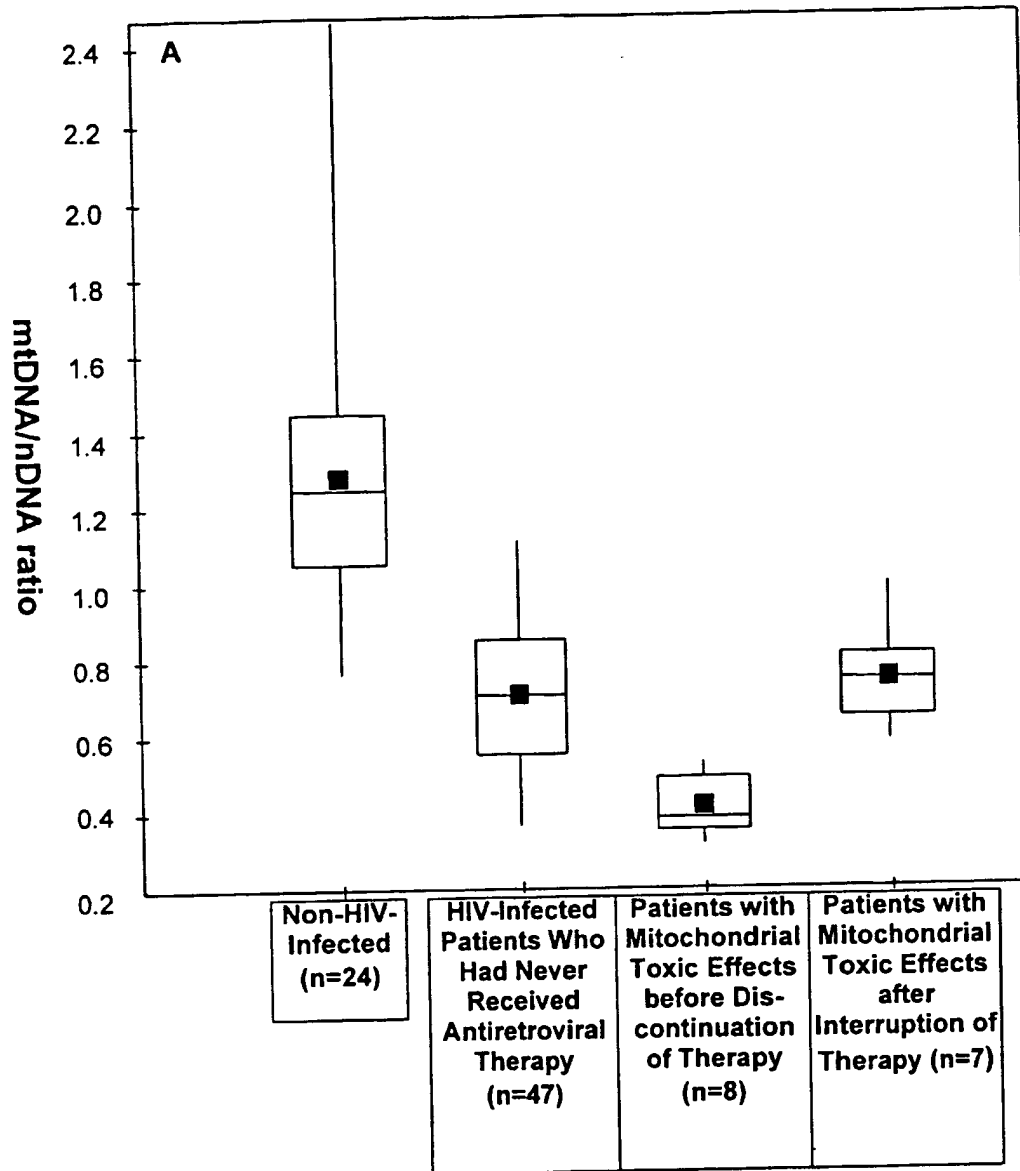


Figure 5



INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 02/00796

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

MEDLINE, EPO-Internal, BIOSIS, WPI Data, PAJ, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 01 35096 A (MITOKOR) 17 May 2001 (2001-05-17) figure 1; example 1	1-8,13, 14,16-19 10-12, 15,20
X	ARNAUDO E ET AL: "Depletion of muscle mitochondrial DNA in AIDS patients with zidovudine-induced myopathy." LANCET. ENGLAND 2 MAR 1991, vol. 337, no. 8740; 2 March 1991 (1991-03-02), pages 508-510, XP001074366 ISSN: 0140-6736	1-5,7,8, 10,12, 14,15
Y	page 508, column 1 -page 510 --/--	9

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☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

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Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
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INTERNATIONAL SEARCH REPORT

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PCT/CA 02/00796

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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